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14. ABSTRACT This work directly addresses how breast cancers grow and how this uncontrolled cellular growth can be stopped. Specifically, we will decipher the role of the 40S ribosome assembly pathway for tumor cell growth and death induced by novel anti-tumor agents. Here we propose to further dissect the role and mechanism of the CK1δ-to-Ltv1 circuit in the maintenance of the malignant state in triple negative breast cancer. In Aim 1 we will clarify if the anti-proliferative activity of CK1δ inhibitors is due to a block of Ltv1 release in ribosome assembly; if the CK1δ-to-Ltv1 circuit is overactive in breast cancer cells; if bypass of the CK1δ-dependent regulation of 40S ribosome assembly augments the tumorigenic potential of cancer cells. In Aim 2 we will confirm preliminary observations that the autophagy and exosome pathways degrade stalled assembling ribosomes, leading to cell death; and test if enhancing these pathways by overexpression or administration of FDA-approved drugs that induce autophagy, potentiates the effect from CK1δ inhibitors.					
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INTRODUCTION

Casein kinase 1 δ (CK1 δ) is a target for cancer drug development, as CK1 δ is amplified and/or overexpressed in several human tumor types, in particular TNBC, and treatment of such tumor cells with CK1 δ inhibitors blocks their growth, survival and tumorigenic potential ¹⁻⁴. Indeed, TNBC are highly sensitive to SR3029, a nanomolar potent and highly selective CK1 δ inhibitor ⁵. CK1 δ is involved in many cellular processes, but in yeast we have shown that its essential cellular role is in the phosphorylation and thus release of the assembly factor Ltv1 from nascent 40S ribosomal subunits ⁶. Deletion of Ltv1 in yeast rescues the lethal effects from CK1 δ knockdown and, excitingly, the efficacy of SR3029 is impaired by the selective knockdown of the ribosome assembly factor Ltv1 in human TNBC ⁶. Nevertheless, while in yeast *Ltv1* was entirely deleted, knockdown of Ltv1 in human cells was incomplete (70-80%), and the deleterious effects from CK1 δ inhibition were not completely rescued ⁶. Thus, it remains unclear whether other targets of CK1 δ contribute to the apoptotic and anti-proliferative effects of SR3029. In this project, we seek to define how CK1 δ regulates TNBC cell growth and survival, and how CK1 δ inhibitors that are currently in preclinical development provoke tumor regression. We will also test the hypothesis that TNBC can develop resistance to CK1 δ inhibitors via phosphomimetic mutations in the Ltv1 phosphorylation site, and we will address the mechanisms by which their activity can be enhanced by co-administration with other drugs that augment the autophagic clearance of ribosome assembly intermediate.

KEYWORDS

- **Triple negative breast cancer**
- **Ribosome assembly**
- **Autophagy**
- **CRISPR/Cas9**

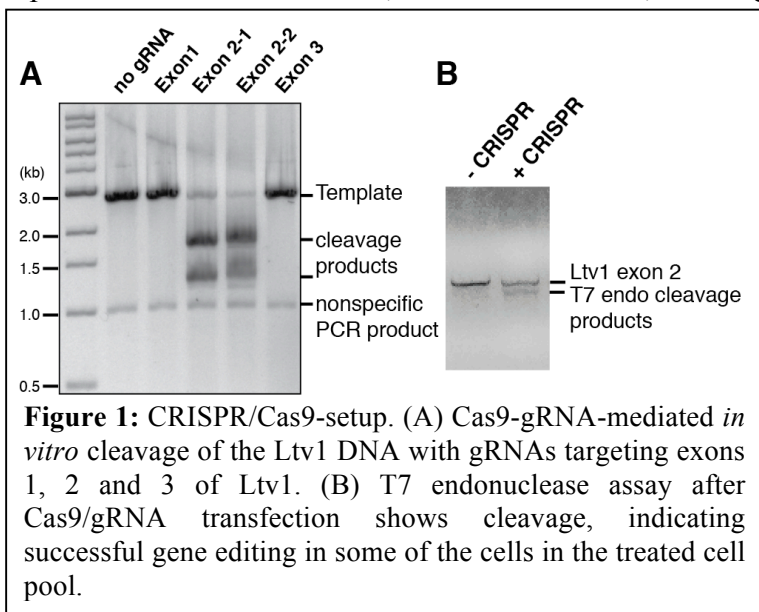
ACCOMPLISHMENTS

Major goals

The major goals in this reporting period were to use the CRISPR/Cas9 technology to produce a cell line where Ltv1 is deleted. This cell line was then to be used to transduce with virus expressing wild type Ltv1, or phosphomimetic Ltv1 mutants, or Ltv1 mutants, bearing phosphorylation-blocked alanine mutants. These will then be assessed for growth defects, defects on ribosome assembly, drug sensitivity etc.

Accomplishments under these goals

To set up the CRISPR/Cas9 experiment, the Karbstein lab first defined optimal targets for the gRNA-mediated endonuclease. Following published methods, we transcribed several guide RNAs, purified recombinant Cas9, and then assayed CRISPR/Cas9



mediated cleavage of a PCR-generated DNA. This demonstrated that the guide RNAs we had designed based on the published methods varied widely in their ability to target Ltv1 DNA, with only 2 out of 4 leading to DNA cleavage (**Figure 1A**). These two guide RNAs, which both target exon 2, were then used in the Karbstein lab in *in vivo* targeting experiments. Unfortunately, this first round of experiments, did not produce a knockout clone, although nearly 100 clones were assayed by Western blotting, and a few initially promising ones were also sequenced.

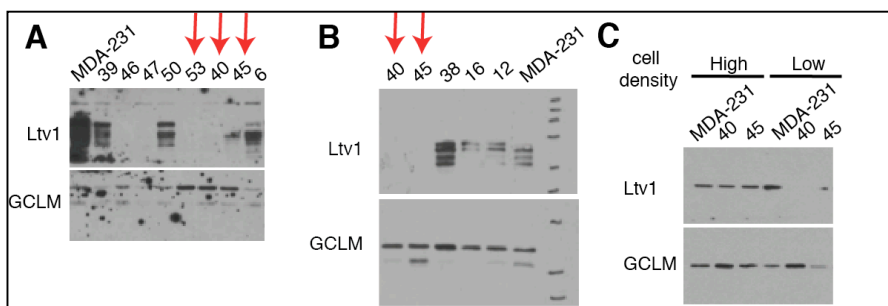


Figure 2: Western blot screen for Ltv1 deletion. (A, B) Early Western blots from clones 40, 45 and 53 indicate loss of Ltv1. (C) After cell expansion Ltv1 expression is recovered, and growing cells to higher density reduces Ltv1 expression in unmodified cells (because ribosome assembly is downregulated), but allows for recovery of Ltv1 expression, perhaps because of a change in the make-up of the culture.

After this first round of experiments, we went back to the drawing board and changed our experimental set-up as follows: instead of transfecting with gRNA-Cas9 complexes (as was recommended to us by the Doudna lab), we transfected a plasmid encoding guide RNAs and nickase Cas9 as described⁷. In addition, we applied

selective pressure by plating cells in the presence of the CK1δ inhibitor SR3029. Initial T7

endonuclease assays on pools of cells indicate successful DNA editing (**Figure 1B**). After serial dilution of these cells to what appeared to be single colonies, several clones were obtained, which were screened by Western blotting followed by sequencing of genomic DNA.

Initial Western analysis of three clones, labeled 40, 45 and 53 indicated that they lacked Ltv1 (**Figure 2**), and genome sequencing showed a loss of sequencing data at the site of the guide RNA (**Figure 3**), seemingly indicating that we had successfully obtained the desired Ltv1 deletion strain.

However, unfortunately, after expansion of the culture, we recovered Ltv1 expression (**Figure 2**). Additionally, growth curves indicate that clones 45 and 53 grow substantially slower than untreated cells, and importantly, also indicate that they are resistant to the CK1 δ inhibitor SR3029 (**Figure 4**). Together, we cautiously interpret these data to indicate that (i) we have produced the desired Ltv1 knockout; (ii) the cells are contaminated, at very low numbers with Ltv1-expressing cells; (iii) Ltv1 deletion confers a substantial grow defect, which allows the Ltv1 containing cells to outgrow the Ltv1 deletion cells, leading to the apparent re-appearance of Ltv1 expression at high cell density.

To confirm our interpretation, we are currently re-diluting our cells to low cell number, and then

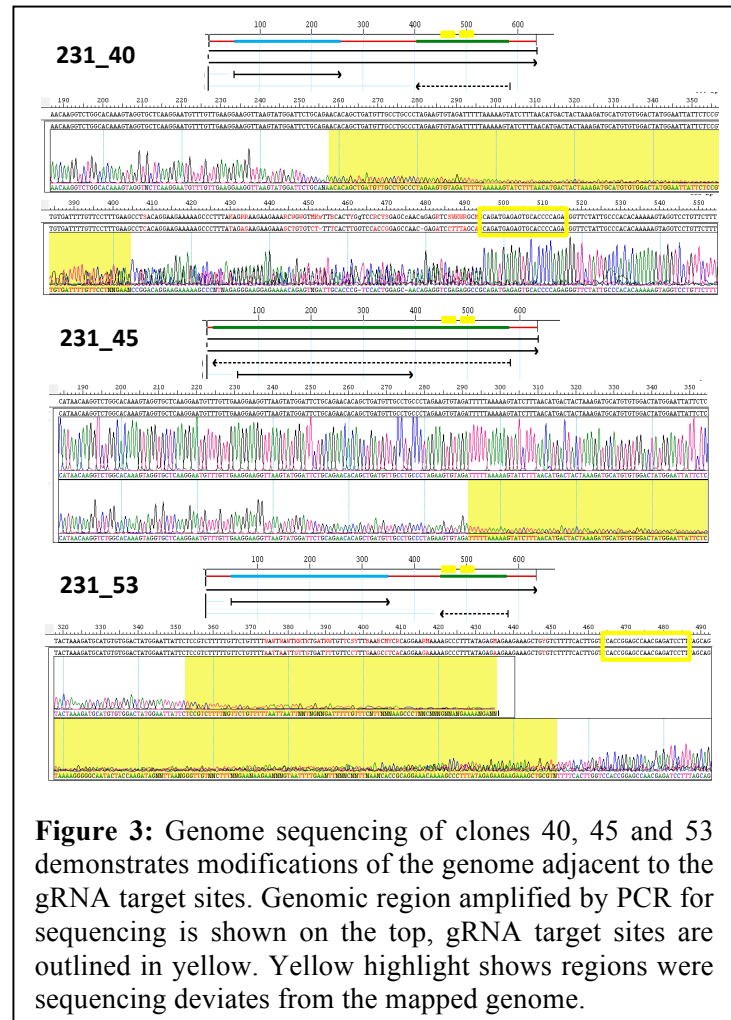


Figure 3: Genome sequencing of clones 40, 45 and 53 demonstrates modifications of the genome adjacent to the gRNA target sites. Genomic region amplified by PCR for sequencing is shown on the top, gRNA target sites are outlined in yellow. Yellow highlight shows regions where sequencing deviates from the mapped genome.

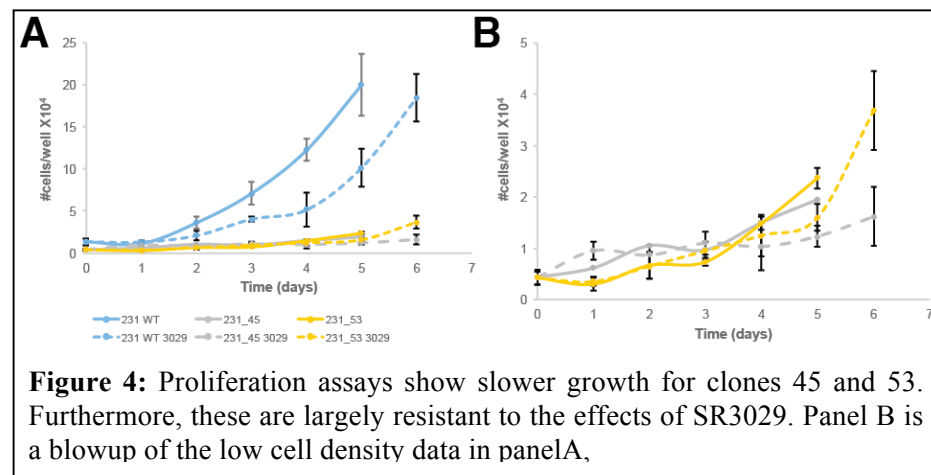


Figure 4: Proliferation assays show slower growth for clones 45 and 53. Furthermore, these are largely resistant to the effects of SR3029. Panel B is a blowup of the low cell density data in panel A,

re-selecting them in the presence of SR3029, this time maintaining them in the drug for a longer time. In addition, the Karbstein lab has produced virus to stably transfect these cells with wild type Ltv1, which is expected to rescue the growth defect, if the

defect truly results from Ltv1 depletion.

Once we have confirmed that we have isolated a stable clone of Ltv1-deleted MDA-231 TNBC cells, we are in a position to quickly to accomplish the remainder of the goals in Major Task1 and 2, as all the reagents for this are in place.

In Major Task 4, the Karbstein lab has moved ahead of schedule to produce Ltv1-GFP vectors, as well as some of the yeast strains.

Training opportunities and professional development

This project has allowed the training of the postdoc in the CRISPR/Cas9 technology.

Dr. Ghalei has also been able to attend the Zing conference on Nucleic Acids.

How were the results disseminated?

Nothing to report, yet.

What do you plan to do during the next reporting period to accomplish the goals?

As described above, the generation of the Ltv1 CRISPR cell line has proved to be more challenging than we had anticipated, partly, because the technique is new to us (and has been used only in one case at Scripps Florida). In addition, the severe growth defect from deletion of Ltv1 in the MDA-231 TNBC cells was somewhat unexpected, as the partial knockdown by siRNAs had only a moderate growth defect⁶, and because the deletion of the yeast gene confers only a moderate growth phenotype. We feel that we have the measures in place to isolate the deletion strain from what we believe is a contaminated mixture as described above. However, it is also possible that we have a heterozygous deletion (sequencing data are unequivocal about genetic changes in the gRNA-targeted region). In that case, it might not be possible to generate a homozygous deletion, if the growth defect does arise from simply the heterozygous deletion. In that case, we will move ahead and carry out the described experiments with the heterozygous deletion, if necessary knocking down Ltv1 expression even more with siRNAs as we have done before⁶.

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- 6 Ghalei, H. *et al.* Hrr25/CK1 δ -directed release of Ltv1 from pre-40S ribosomes is necessary for ribosome assembly and cell growth. *Journal of Cell Biology* **208**, 745-759 (2015).
- 7 Ran, F. A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat Protoc* **8**, 2281-2308, doi:10.1038/nprot.2013.143 (2013).

IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report, yet.

What was the impact on other disciplines?

Nothing to report, yet.

What was the impact on technology transfer?

Nothing to report, yet. But Dr. Roush is commercializing SR-3029, and therefore, this research is anticipated to make an impact in the future.

What was the impact on society beyond science and technology?

Nothing to report, yet.

CHANGES/PRODUCTS

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

As described in detail above, the generation of the Ltv1-knockout strain is taking substantially longer than expected, likely because this strain is so growth disadvantaged that it gets lost in a larger population of unedited cells. We have and are further implementing several strategies to ameliorate the growth differences between edited and unedited cells. In addition, we are establishing other back up plans to investigate the drug sensitivity and ribosome assembly phenotypes of the cultures we currently have as well as potential heterozygous populations.

Changes that had a significant impact on expenditures

Significant changes in use or care of human subjects

N/A

Significant changes in use or care of vertebrate animals.

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

PRODUCTS

Nothing to report, yet.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	<i>Katrin Karbstein</i>
Project Role:	<i>PI</i>
Researcher Identifier (Era commons ID)	<i>kkarbst</i>
Nearest person month worked:	<i>2</i>
Contribution to Project:	<i>Dr. Karbstein supervises the effort in the Karbstein lab.</i>
Funding Support:	

Name:	<i>Homa Ghalei</i>
Project Role:	<i>Research Associate (Postdoc)</i>
Researcher Identifier (e.g. ORCID ID):	<i>hghalei</i>
Nearest person month worked:	<i>12</i>
Contribution to Project:	<i>Dr. Ghalei has carried out the CRISPR deletions.</i>
Funding Support:	

Name:	<i>Joanne Doherty</i>
Project Role:	<i>Staff Scientist</i>
Researcher Identifier (e.g. ORCID ID):	<i>jrdoherty</i>
Nearest person month worked:	<i>3</i>
Contribution to Project:	<i>Dr. Doherty has assisted in the creation of CRISPR deletion strains.</i>
Funding Support:	<i>Florida Brain Cancer Initiative (to Dr. Doherty), and HHMI (to Dr. Karbstein)</i>

Name:	<i>John Cleveland</i>
Project Role:	<i>PI</i>
Researcher Identifier (e.g. ORCID ID):	<i>KKARBST</i>
Nearest person month worked:	<i>2</i>
Contribution to Project:	<i>Dr. Cleveland supervises the effort in the Cleveland lab.</i>
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Since the submission of the JIT, the Karbstein lab was awarded a second R01 for her work on ribosome assembly. This funding is to investigate the assembly of the mRNA entry channel in yeast, using a combination of structural and biochemical assays.

In addition, Dr. Karbstein was named an HHMI Faculty Scholar.

What other organizations were involved as partners?

Nothing to report.

SPECIAL REPORTING REQUIREMENTS

CLEVELAND ANNUAL REPORT: DOD_W81XWH-15-BCRP-BREAKTHROUGH-FL12

1. INTRODUCTION

The first-in-kind DOD-funded Breakthrough collaborative studies of the laboratories of Dr. Katrin Karbstein (Initiating Principal Investigator [PI], Scripps-Florida) and Dr. John L. Cleveland (Collaborating/Partnering PI, Moffitt Cancer Center) seeks to validate targeting 40S ribosome assembly as a therapeutic target for aggressive breast cancer subtypes, including triple negative breast cancer (TNBC) that currently lacks targeted therapies. Specifically our research team has shown that the serine/threonine casein kinase-1 δ (CK1 δ) phosphorylates the 40S ribosome assembly factor Ltv1 in both yeast and TNBC cells, and that selective knockdown or silencing of CK1 δ , or forced expression of Ltv1 mutant that cannot be phosphorylated by CK1 δ , blocks ribosome assembly in yeast and compromises the growth and survival of TNBC cells. Further, we have shown that forced overexpression of a phosphor-mimetic Ltv1 mutant (i.e., a constitutively active mutant) can override the deleterious effects of CK1 δ inhibition or silencing on ribosome assembly. Thus, these DOD funded studies suggest that the CK1 δ -to-Ltv1 circuit is a tractable vulnerability for TNBC and for other breast cancers that we have shown overexpress CK1 δ such as HER2+ and luminal B breast cancer subtypes. Accordingly our DOD supported studies seek to validate this circuit as a therapeutic target for TNBC so these findings can be advanced into the breast oncology clinic. Our studies include those that seek to further understand the regulation and role of this circuit in TNBC breast cancer growth and survival, and to assess if TNBC regression that is triggered by treatment with CK1 δ inhibitors involves defects in ribosome assembly. We will also test if TNBC tumors can develop resistance to CK1 δ inhibitors, if such resistance involves the acquisition of gain-of-function phosphomimetic mutations in Ltv1, and if such resistance can be overcome with other drugs that augment destruction ribosome assembly intermediates via the autophagy pathway.

2. KEYWORDS

- Triple negative breast cancer
- CK1 δ -to-Ltv1 circuit
- Ribosome assembly
- Breast cancer therapy
- Autophagy

3. ACCOMPLISHMENTS

Major Goals and Accomplishments

Given the clear evolutionary selection against CRSIPR/cas9-directed *Ltv1*-deficient TNBC generated by the Karbstein lab (see accompanying report), which are needed to evaluate the effects of phosphorylation defective and phosphor-mimetic Ltv1 mutants on TNBC growth and survival and on the effects of CK1 δ inhibitors on these cells, we were not able, at this juncture, to transduce these *Ltv1* knockout cell lines with existing Dox-inducible (pLKO-Tet-on) lentiviruses that will allow for conditional expression of Ltv1, Ltv1-A and Ltv1-D transgenes. Once these hurdles have been overcome, by selection for the slow-growing Ltv1-deficient clones in the presence of the CK1 δ inhibitor SR-3029 (see accompanying report) we will transduce these cells with these lentiviruses bearing these Ltv1 transgenes and assess effects of wild type and mutant Ltv-1 on growth, clonogenicity (growth in soft agar), survival, migration and invasion ex vivo, and on tumorigenic potential in vivo using orthotopic (cleared mammary fat pad) xenograft studies. The sensitivity of these 3 Ltv1 cohorts of TNBC to top CK1 δ inhibitors will also be assessed.

An important goal of our research team is to test if TNBC tumors can develop resistance to CK1 δ inhibitors, and if such resistance involves the acquisition of gain-of-function phosphomimetic mutations in Ltv1 or other compensatory gain-of-function mutations that would rescue ribosome assembly. To this end, by serially transplanting surviving MDA-MB-231 TNBC cells that were first cultured in the presence of EC₅₀ doses of the CK1 δ inhibitor SR-3029 (in 6-well plates) and then sequentially increasing the doses of the SR-3029, we were able to derive several independent MDA-MB-231-SR-3029-resistant (MDA-MB-231 SRR) cell lines (**Figure 1**). To assess the generality of our findings similar studies generating SR-3029 resistant lines have been performed in other tumor systems (e.g. melanoma and multiple myeloma) that are also highly sensitive to this CK1 δ inhibitor.

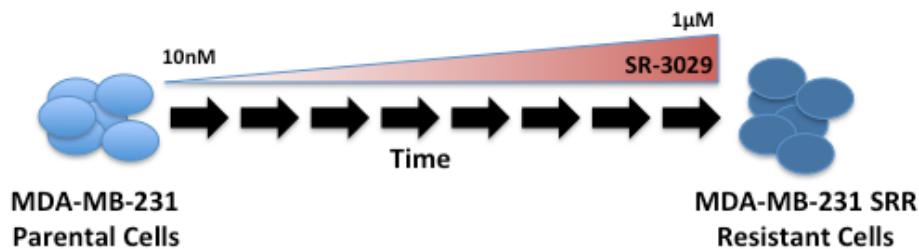


Figure 1. Experimental design for the generation of SR-3029-resistant MDA-MB-231 TNBC cells.

MDA-MB-231 SRR resistant cells were then re-tested for their sensitivity of SR-3029 by

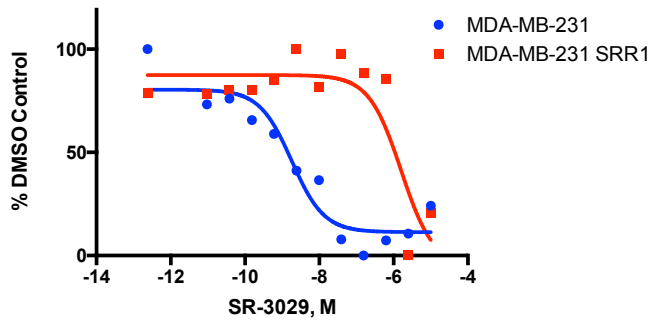


Figure 2. MTT assay of SR-3029-resistant vs. parental MDA-MB-231 cells at the indicated doses of SR-3029

performing a standard MTT proliferation assay. As predicted these resistant cells remained refractory to doses of SR-3029 that completely blocked the proliferation of parental MDA-MB-231 cells (**Figure 2**).

To further characterize SR-3029 resistant tumor cells we compared

their rates of proliferation using standard growth assays. Notably,

In TNBC, CK1 δ inhibition or silencing disables WNT- β -catenin signaling and the expression of β -catenin/TCF target genes. We therefore isolated RNA from parental and SR-3029 resistant tumor cells and compared their expression of WNT- β -catenin pathway genes using a Nanostring assay of 180 genes in this pathway. Notably, SR-3029 resistant tumor cells displayed markedly different expression of WNT- β -catenin pathway genes than parental tumor cells and the two resistant derivatives also had significant differences in these genes (**Figure 4**); These findings support the notion that there is more than one pathway that can lead to resistance to CK1 δ inhibitors and that the selection for resistance does not appear to include events that totally override the inhibitory effects of SR-3029 on the expression WNT- β -catenin pathway genes. We are currently performing RNA-seq analyses of parental versus SR-3029 resistant tumor cells and will assess effects on the expression of Ltv-1 and

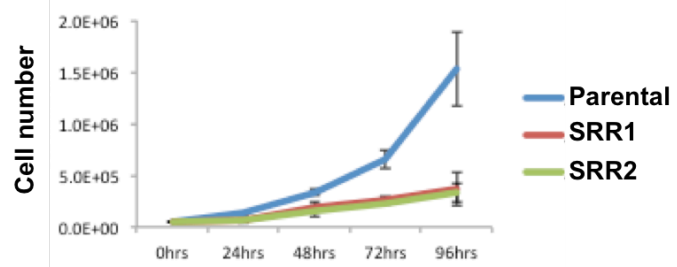


Figure 3. Growth curves were performed with parental vs. SR-3029-resistant tumor cell line and cell counts were performed at the indicated intervals

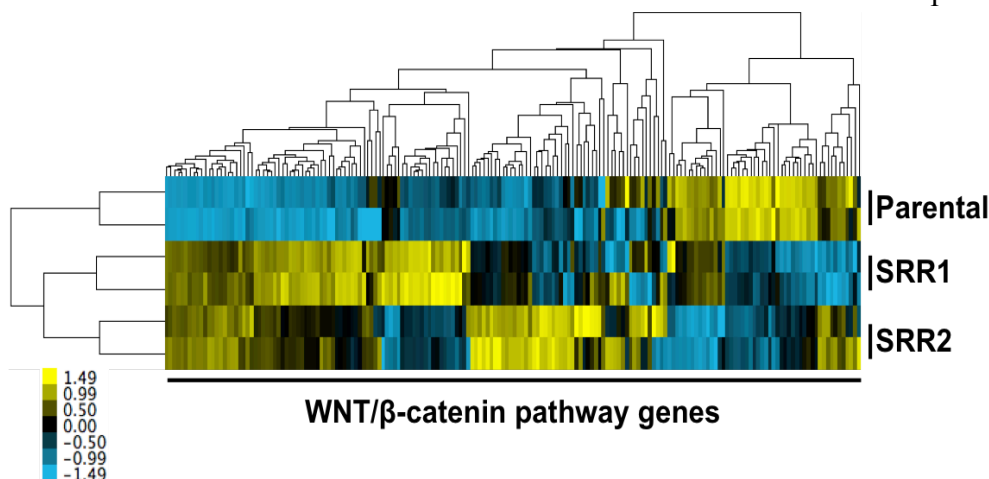


Figure 4. The expression of WNT/ β -catenin pathway genes is altered in SR-3029 resistant tumor cells. Nanostring analysis of WNT/ β -catenin pathway genes in the indicated tumor cell lines is shown. Genes in the pathway that are up-regulated are in yellow; those that are repressed are in blue.

other ribosome assembly factors.

At this juncture all of our TNBC cell studies, have relied on a

single inhibitor (SR-3029) of CK1 δ including those showing the gain-of-function Ltv1-D mutant can override the effects of the inhibitor on ribosome assembly. We have therefore screened additional new CK1 δ inhibitors obtained from our collaborator Dr. William Roush for their anti-tumor activity in MTT assays. Notably several of these new CK1 δ inhibitors (which have been proven as selective CK1 δ kinase inhibitors using in vitro kinase assays) have improved potency in MTT assays (**Figure 5**). These inhibitors will be used to validate the effects of the SR-3029 inhibitor on ribosome assembly and Ltv-1 phosphorylation, and TNBC that are resistant to these inhibitors will also be generated, to determine if resistance is associated with gain-of-function mutations in Ltv1 or in other factors or regulators of ribosome assembly.

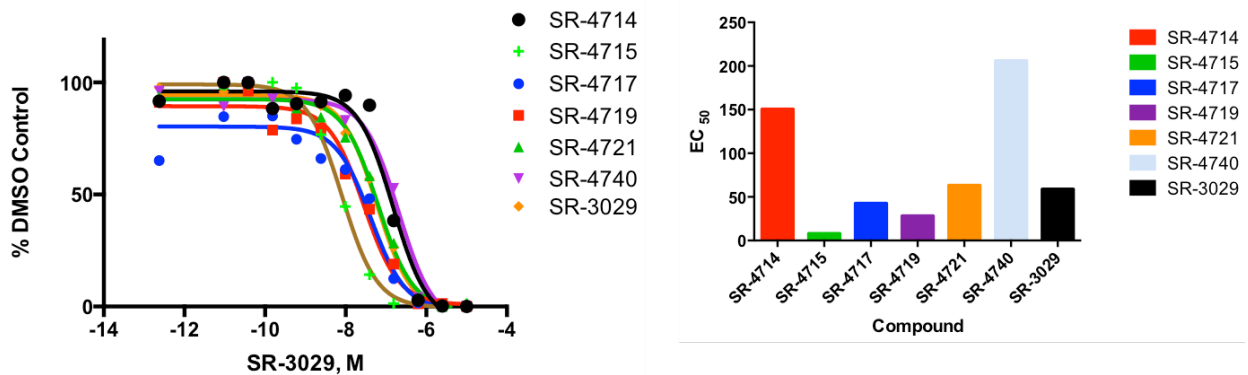


Figure 5. Anti-tumor potency of novel, 3rd generation CK1 δ inhibitors. *Left*, EC₅₀ dose response curves with the indicated CK1 δ inhibitors. *Right*, EC₅₀ values of the new CK1 δ inhibitors vs. SR-3029 (black bar).

Training Opportunities and Professional Development

These DOD funded studies allowed for training of the postdoctoral fellow in Nanostring technologies and in developing drug resistant tumor cells.

How were the results disseminated?

At this juncture our studies have not been published.

Plans for next reporting period

I. Perform Remaining experiments for Major Task1, and the Studies of Major Task 2 and Major Task 3

(i) CRISPR/cas9-deleted Ltv1-null TNBC clones that have been purified by Dr. Karbstein by growth in the presence of the CK1 δ inhibitor SR-3029 (see accompanying report) will be transduced with pLKO-Tet-On-GpNLuc lentiviruses that harbor the reverse tetracycline transactivator (rtTA²) and that are engineered to express wild type Ltv1, Ltv1-A, or Ltv1-D transgenes along with the imaging reporter GpNLuc that was created by the Cleveland lab (an in-frame fusion of eGFP and the small subunit of Nano-luciferase, NLuc). Infected cells will be isolated by FACS for eGFP, and effects of wild type and mutant Ltv-1 on growth, clonogenicity (growth in soft agar), survival (apoptosis assays), migration and invasion, and ribosome assembly (with Dr. Karbstein) ex vivo will be assessed +/- Dox (250 μ g/ml, to induce the Ltv1 transgenes). Efficient expression of the Ltv-1 transgenes will be verified by

immunoblots and qRT-PCR. The sensitivity of these cells to SR-3029 and other top CK1δ inhibitors will also be assessed in cultures +/- Dox.

(ii) We will assess the tumorigenic potential of Ltv1-deficient vs. replete MDA-MB-231 TNBC, as well as Ltv1-deficient cell engineered to inducibly re-express wild type Ltv1, Ltv1-A, or Ltv1-D in vivo using orthotopic (cleared mammary fat pad) xenograft studies. Following transplant half of each of these cohorts will be switched to Dox chow to inducibly express the Ltv1 transgenes. Effects on tumor progression will be evaluated by switching to Dox chow 7 days after transplant. Effects on tumor regression will be monitored in cohorts switched to Dox chow when tumor reach 100-mm³. The sensitivity of these 3 Ltv1 cohorts of TNBC to top CK1δ inhibitors will also be assessed.

II. Fully characterize CK1δ inhibitor-resistant breast cancer cells

(i) Assess resistant TNBC cells for expression/mutation of Ltv1, and for alterations in ribosome assembly factors. Assess ribosome assembly intermediates in these cells with Dr. Karbstein. Repeat these analyses in an additional TNBC cell line, and with a second CK1δ inhibitor. Assess changes in expression via RNA-seq, perform pathway analyses and confirm select differentially expressed genes (e.g., those involved in ribosome assembly) by qRT-PCR, and assess effects on protein levels by immunoblots analyses.

(ii) Assess biological properties (growth, apoptosis, invasion and migration) and tumorigenic potential of CK1δ inhibitor-resistant vs. parental TNBC +/- SR-3029 treatment.

4. IMPACT

What was the impact on the development of the principal discipline(s) of the project?

Our findings support the hypothesis that Ltv1 represents a critical target that when disabled markedly compromises the growth of TNBC cells.

What was the impact on other disciplines? Our analyses of tumor cell lines resistant to CK1δ inhibitors will inform strategies for overcoming resistance, which could be applied to other tumor types treated with such agents.

What was the impact on technology transfer? To date there has been no technology transfer. However, CK1δ inhibitors are being further developed by our collaborator Dr. Roush to produce clinically suitable safety assessment candidates.

What was the impact on society beyond science and technology? Nothing to report at this juncture.

5. CHANGES/PRODUCTS

Changes in approach and reasons for change. Nothing to report at this juncture.

Actual or anticipated problems or delays and actions or plans to resolve them

As noted above, and in the accompanying report of Dr. Karbstein, the generation of the Ltv1-knockout strain of MDA-MB-231 TNBC cells using CRISPR/Cas9 technology proved difficult, as our hypothesis was correct, where Ltv1 is essential for proper growth of TNBC. In particular the very-slow-growth phenotypes of clear Ltv1-null cells is out competed by very minor unedited (i.e., Ltv1 wild type) cells that are still in the culture. However, such Ltv1-null clones are (as would be predicted if Ltv1 were *the* key target) intrinsically resistant to the CK1 δ inhibitor SR-3029, allowing us to now select for and grow these cells and kill off wild-type Ltv1 cells. Thus, as soon as these cells are fully purified we are poised to perform the remaining studies of Major Task 1, as well as those of Major Tasks 2 and 3. We will also evaluate the phenotypes of Ltv1^{+/-} TNBC cells, to see if there are effects of *Ltv1* heterozygosity of breast cancer cell growth, survival, invasion and migration, ribosome assembly and tumorigenic potential +/- treatment with SR-3029.

Changes that had a significant impact on expenditures. Nothing to report at this juncture.

Significant changes in use or care of human subjects. Not applicable.

Significant changes in use or care of vertebrate animals. Nothing to report.

Significant changes in use of biohazards and/or select agents. Nothing to report.

6. PRODUCTS

Nothing to report at this juncture.